# Appendix A

# INTERLEUKIN-12 : A NOVEL HETERODIMERIC CYTOKINE WITH POTENTIAL ANTITUMOR APPLICATIONS

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## *Abstract*

A number of experimental and human tumors, including human gliomas, have been shown to possess a defect in immunogenicity which can be overcome by the administration of certain cytokines. We have recently identified a novel heterodimeric cytokine which has been designated interleukin-12. This cytokine can enhance both specific cytolytic T lymphocyte responses and nonspecific LAK cell responses, as well as elicit the production of cytostatic/cytotoxic cytokines such as interferon-γ and tumor necrosis factor. These properties suggest that IL-12 may have potential utility in the treatment of cancer.

*Key words*: interleukin-12, cytokine, LAK cells, cytolytic T lymphocytes, interferon-γ

## *Introduction*

Previous studies on cell-mediated immune responses to cultured human gliomas revealed several properties of human glioma cells which may contribute to their ability to escape cell-mediated immune destruction (5). These include (i) a defect in immunogenicity which can be overcome by the addition of certain cytokines (5-7), (ii) the secretion of immunosuppressive substances (5, 19), and (iii) the production of hyaluronic acid-containing cell coats which constitute a physical barrier to contact between lymphocytes and glioma cells (2, 4, 5). It was our interest in the first of these escape mechanisms and in delineating cytokines which could facilitate human cytolytic T lymphocyte responses to allogeneic glioma and melanoma cells *in vitro* that led us to identify a new cytokine which has recently been designated interleukin-12 (IL-12) (10, 11, 20). The same cytokine was identified by Kobayashi *et al.* (12, 21) on the basis of its ability to induce interferon-γ (IFN-γ) production by human peripheral blood mononuclear cells (PBMC) in culture.

Interleukin-12, which was originally called cytotoxic lymphocyte maturation factor (20) or natural killer cell stimulatory factor (12), is a heterodimeric cytokine composed of disulfide bonded subunits having molecular masses of 35 kDa and 40 kDa,

respectively (12, 20). IL-12 was originally purified from conditioned media from cultures of activated human B lymphoblastoid cells. The cDNA encoding each of the two subunits of IL-12 has recently been cloned, and coexpression of both subunit cDNAs in COS cells was shown to result in the secretion of biologically active IL-12 (11, 21). Expression of either subunit alone did not result in the production of bioactive cytokine. Studies on purified B cell-derived IL-12 and on recombinant IL-12 have demonstrated that IL-12 mediates several biological activities which make it an attractive candidate for testing as an antitumor agent. Specifically, IL-12 can (i) enhance both specific cytolytic T lymphocyte (CTL) responses and nonspecific lymphokine-activated killer (LAK) cell responses, (ii) act as a growth factor for both activated T cells and natural killer (NK)/LAK cells, and (iii) induce the secretion of cytostatic/cytotoxic cytokines such as IFN-γ and tumor necrosis factor (TNF). Experiments demonstrating these activities of IL-12 are summarized herein.

## *Materials and Methods*

Cytokines. Human IL-12 was produced by activation of NC-37 B lymphoblastoid cells with PMA plus calcium ionophore A23187 and was purified as described in reference 20. The NC-37-derived IL-12 used in these studies was >95% pure as assessed by SDS-PAGE. Recombinant IL-12 (rIL-12) was produced by cotransfection of COS cells with a 1:1 molar ratio of the two subunit cDNAs of IL-12 as described in reference 11. Crude supernatant fluid from cultures of doubly transfected cells was used as a source of rIL-12 in these experiments. Purified human rIL-2 was supplied by Dr. F. Khan, Department of Bioprocess Development, Hoffmann-La Roche Inc.

NK and LAK cell activation assays. PBMC were isolated from normal donors as previously described (20). NK cell activation assays were performed by incubating unfractionated PBMC (4 x $10^6$/ml) overnight at 37°C in the indicated concentrations of IL-12. On the following day, the cells were harvested and assayed for lytic activity on $^{51}Cr$-labeled Daudi cells in a 5 hr $^{51}Cr$ release assay as previously described (20). The percent specific $^{51}Cr$ release was calculated as $|(e - c)/(100 - c)| \times 100$, where e is the percentage of $^{51}Cr$ released from target cells incubated with lymphocytes and c is the percentage of $^{51}Cr$ released spontaneously from target cells incubated alone. For LAK activation assays, low density lymphocytes were isolated from PBMC by removal of accessory cells using

glutamic acid dimethyl ester followed by centrifugation over a discontinuous Percoll gradient as described (22). Low density lymphocytes ($10^6$/ml) were cultured at 37°C for 4 days with the indicated amounts of IL-12 and/or IL-2, after which the cells were harvested and tested for their lytic activity on Daudi target cells.

CTL generation assays. High density lymphocytes were isolated from PBMC by accessory cell depletion followed by Percoll gradient centrifugation as previously described (22). High density lymphocytes ($10^6$/ml) were incubated at 37°C in 1 ml cultures with γ-irradiated (10,000 rad) HT144 melanoma cells (5 x $10^4$/ml). Ten μM hydrocortisone sodium succinate was included in each culture to minimize the generation of nonspecific LAK cells (8). After 6 days, the lymphocytes were harvested from the wells and tested for their ability to lyse $^{51}$Cr-labeled HT144 melanoma cells or, as a specificity control, $^{51}$Cr-labeled K562 cells in an overnight $^{51}$Cr release assay as described (22). One lytic unit was defined to be the number of lymphocytes required to cause 30% specific $^{51}$Cr release.

Lymphocyte proliferation assays. The proliferation of PHA-activated PBMC or resting PBMC in response to IL-12 and/or IL-2 was assessed by $^3$H-thymidine incorporation as described (10, 20).

Interferon induction assay. For induction of IFN-γ secretion, unfractionated PBMC ($10^7$/ml) were cultured overnight at 37°C with the indicated concentrations of IL-12 and/or rIL-2. On the following day, the supernatant fluids were harvested from each culture and tested for the presence of IFN-γ by means of a cytopathic effect inhibition assay using human amniotic WISH cells and vesicular stomatitis virus as described (8).

## *Results*

IL-12 enhances both nonspecific NK/LAK cell responses and specific CTL responses. We have previously reported that in cultures containing hydrocortisone, IL-12 synergized with IL-2 in the induction of human LAK cell responses *in vitro* (11, 20); however IL-12 by itself was inactive. Hydrocortisone was included in those assays to inhibit endogenous cytokine production and the triggering of cytokine cascades so that the effects of individual added cytokines could be unambiguously studied. However, when similar experiments were performed in which hydrocortisone was omitted from the assay, IL-12 by itself was found capable of enhancing the lytic activity of NK/LAK cells (Fig. 1). In agreement with the results of Kobayashi *et al.* (12),

overnight culture of PBMC with IL-12 resulted in enhanced NK lytic activity (Fig. 1A). Likewise, culture of unseparated PBMC (data not shown) or low density peripheral blood lymphocytes (Fig. 1B) with IL-12 for 4 days resulted in the generation of nonspecific LAK cell activity (Fig. 1B). The IL-12-induced LAK cell activity in these cultures was mediated predominantly by $CD56^+$ NK cells (data not shown). In both the overnight NK activation assay and the 4-day LAK cell induction assay, IL-12 was active at lower pM concentrations than IL-2 (12 and M. Gately, unpublished results). However, in both assays the maximum lytic activity which could be generated in the presence of optimal concentrations of IL-12 was lower than the maximum lytic activity induced by an optimal amount of IL-2 (Fig. 1). On the average, the maximum IL-12-induced lytic activity was approximately 25-30% of the maximum IL-2-induced lytic activity.

The observation that IL-12-mediated LAK cell induction did

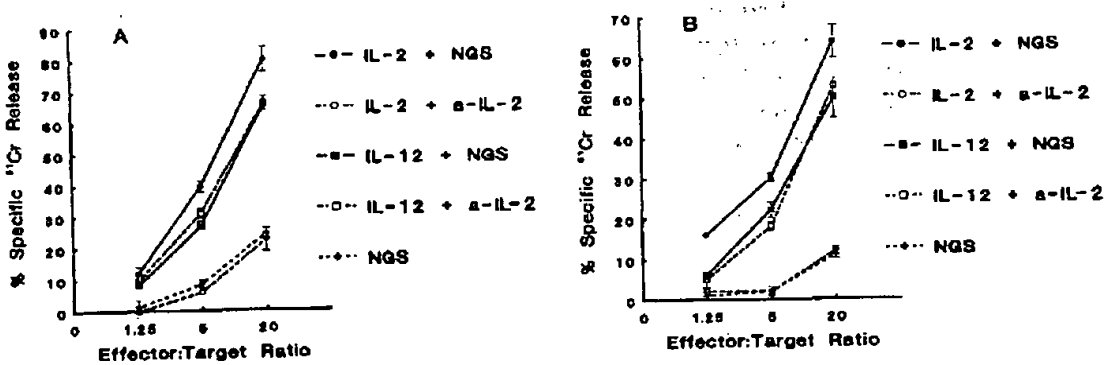


Figure 1. IL-12 activates NK cells in an overnight assay (A) and LAK cells in a 4-day assay (B) independent of IL-2. In (A), unfractionated PBMC were cultured overnight in the presence of 50 U/ml rIL-2 or 50 U/ml purified NC-37-derived IL-12 with goat anti-human IL-2 (anti-IL-2) at 1/500 final dilution or control normal goat serum (NGS). The following day, the cells were harvested and assayed for their ability to lyse $^{51}$Cr-labeled Daudi cells at the indicated effector:target ratios. In (B), low density human peripheral blood lymphocytes were cultured with 4 U/ml rIL-2 or 50 U/ml purified NC-37-derived IL-12 plus 1/1000 anti-IL-2 or NGS for 4 days and then tested for their lytic activity on Daudi targets. The indicated values are the means of quadruplicates, and the error bars indicate 1 standard error of the mean. For points without error bars, the error fell within the symbol (< ± 1%).

not occur in the presence of hydrocortisone (11, 20) suggested that IL-12-mediated induction of LAK cells may depend on the *in situ* production of other cytokines. Studies using a potent neutralizing goat antiserum to human IL-2 indicated that IL-12-mediated activation of NK/LAK cells in either the overnight or the 4-day assay was independent of IL-2 (Fig. 1). However, in other studies we found that IL-12-induced generation of LAK cell activity in the 4 day assay could be partially inhibited by an antiserum against human TNF-α, suggesting that IL-12-mediated LAK cell activation is partially dependent upon the *in situ* production of TNF-α (M. Gately, A. Wolitzky, P. Quinn *et al.*, manuscript in preparation). This possibility is consistent with the observation that IL-12 can induce the secretion of TNF-α (see below).

In addition to its ability to enhance the nonspecific lytic activity of NK/LAK cells, IL-12 was also found to facilitate specific CTL responses to weakly immunogenic, allogeneic melanoma cells in culture (Fig. 2). In these experiments, the addition of IL-12 to cultures of high density peripheral blood lymphocytes and γ-

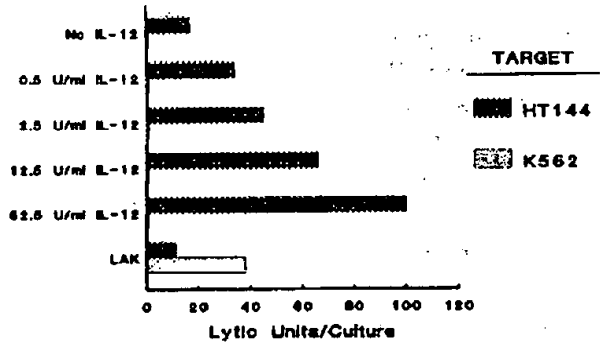


**Figure 2.** IL-12 causes dose-dependent enhancement of a specific CTL response to an allogeneic melanoma in culture. High density peripheral blood lymphocytes were cultured with γ-irradiated, allogeneic HT144 melanoma cells in the indicated concentrations of rIL-12. After 6 days, the cells were harvested and tested for their ability to lyse $^{51}$Cr-labeled HT144 melanoma cells or, as a specificity control, $^{51}$Cr-labeled K562 cells. LAK cells were generated by culturing low density peripheral blood lymphocytes in 5 U/ml rIL-2 without hydrocortisone and were included in the lytic assay to demonstrate the relative sensitivity of the two target cell lines to nonspecific LAK cell-mediated lysis.

irradiated allogeneic HT144 melanoma cells caused a dose-dependent increase in the generation of lymphocytes which specifically lysed the melanoma cells. The specificity of lysis was indicated by the failure of these lymphocyte populations to lyse cells of the K562 erythroleukemia line, a cell line which is more sensitive to LAK cell-mediated nonspecific lysis than is the HT144 melanoma line (Fig. 2). Moreover, lysis of HT144 melanoma targets in this type of assay was shown to be mediated by $CD3^+$ T lymphocytes (M. Gately and A. Wolitzky, unpublished results). Culture of high density lymphocytes in IL-12 in the absence of irradiated melanoma cells did not result in the generation of lytic effector cells (data not shown). Unlike IL-12-induced LAK cell activation, IL-12-mediated enhancement of specific CTL responses could be completely abolished by anti-IL-2 (M. Gately, A. Wolitzky, P. Quinn, *et al.* manuscript in preparation). Hence, IL-12 can induce the activation of nonspecific NK/LAK cells by an IL-2-independent mechanism and enhance specific CTL responses by a mechanism which requires the *in situ* production of IL-2.

IL-12 is a growth factor for activated T and NK cells. In addition to its ability to enhance the lytic activity of T and NK cells, IL-12 can stimulate the proliferation of both activated T and NK cells. IL-12 was shown to cause a dose-dependent enhancement in the proliferation of PHA-activated peripheral blood lymphoblasts (Fig. 3), which were greater than 90% $CD3^+$ T cells. Experiments

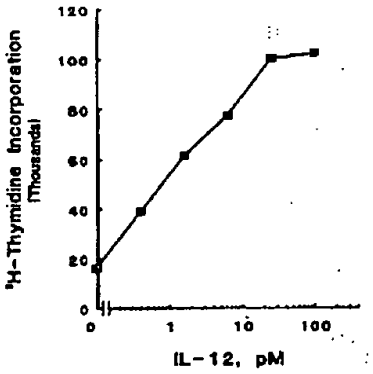


Figure 3. Dose-response for stimulation of proliferation of human PHA-activated lymphoblasts by purified, NC-37-derived IL-12. Proliferation was measured in a 48-hr assay as described in reference 20. One pM purified, NC-37-derived IL-12 is equal to 6 U/ml.

with isolated subsets of T cells and with cloned T cell lines showed that activated T cells of both the $CD4^+$ and $CD8^+$ subsets could proliferate in response to IL-12 (10). Moreover, IL-12 also stimulated the proliferation of IL-2-activated $CD56^+$ NK cells (10). The concentration of IL-12 required to stimulate half-maximum proliferation of activated T or NK cells was 1-8 pM, approximately 6-fold lower than the concentration of IL-2 which caused half-maximum proliferation. However, the maximum proliferation induced by IL-12 was only about 50% of the maximum proliferation elicited by optimal concentrations of IL-2 (10).

Unlike IL-2, IL-12 elicited little or no proliferation of resting peripheral blood lymphocytes (10). However, in cultures containing suboptimal concentrations (1-25 U/ml) of IL-2, IL-12 enhanced the IL-2-induced proliferation of resting peripheral blood lymphocytes when proliferation was measured after 7 to 10 days (Fig. 4). $CD56^+$ NK cells were shown to be the predominant lymphocyte subpopulation proliferating in these cultures (10). The mechanism by which the combination of IL-2 and IL-12 caused synergistic proliferation in these experiments likely involved both IL-12-induced prolongation of IL-2 receptor expression and IL-2-induced upregulation of IL-12 receptor expression so that the responsiveness of the NK cells to both cytokines was enhanced (10).

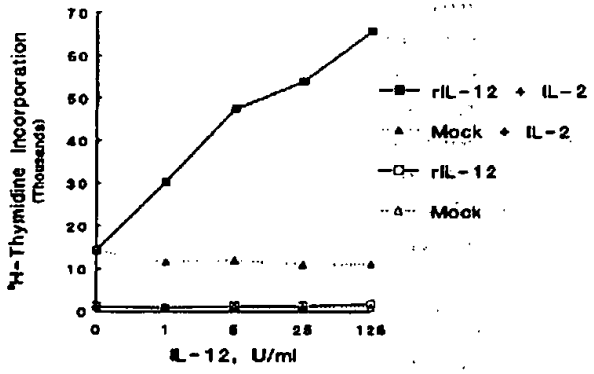


Figure 4. IL-12 enhances IL-2-dependent proliferation of resting PBMC. Resting human PBMC were cultured in the indicated amounts of human rIL-12 or an equivalent volume of control mock transfected COS culture supernatant fluid. In addition, 5 U/ml human rIL-2 was present in some cultures, as indicated. $^3$H-thymidine incorporation was measured after 7 days. The culture conditions were as described in reference 10.

IL-12 induces the production of other cytokines by resting PBMC. Kobayashi *et al.* (12) first reported that IL-12, both alone and in synergy with IL-2, could induce the production of IFN-γ by resting PBMC. We have confirmed these results (Table 1). Chan *et al.* (1) demonstrated that the induction of IFN-γ by IL-12 was

Table 1

IL-12 Synergizes with IL-2 to Induce Interferon-γ Production by Peripheral Blood Mononuclear Cells[a]

| IL-12 | IL-2 | Interferon-γ Titer (U/ml) | |
|---|---|---|---|
| U/ml | U/ml | Expt. 1 | Expt. 2 |
| 0 | 0 | < 80 | < 40 |
| 500 | 0 | 2,560 | |
| 100 | 0 | 960 | < 40 |
| 20 | 0 | | < 40 |
| 0 | 10 | 80 | < 40 |
| 500 | 10 | 10,240 | |
| 100 | 10 | 7,680 | 640 |
| 20 | 10 | 5,120 | 640 |

[a]Human PBMC were cultured overnight with the indicated concentrations of purified, NC-37-derived IL-12 and/or rIL-2. The culture supernatant fluids were tested for interferon activity in an antiviral assay as described in Materials and Methods. Antibody neutralization studies confirmed that the interferon activity detected in these supernatants was due to IFN-γ. IL-12 itself was inactive in the antiviral assay. The PBMC used in experiments 1 and 2 were from two different donors.

accessory cell dependent and that both T and NK cells could produce IFN-γ in response to IL-12. Recently, it has been shown that IL-12 can elicit the production of TNF-α from isolated NK cells (B. Naume, M. Gately, and T. Espevik, manuscript in preparation). However the amount of TNF produced in response to IL-12 was < 1/10 the amount of TNF that was secreted in response to IL-2.

## *Discussion*

There exist several different mechanisms which the immune system may employ to destroy tumor cells. These include the generation of CTL capable of recognizing and destroying tumor cells bearing specific tumor antigens, the nonspecific lysis of tumor cells by NK/LAK cells and by activated monocytes, the production of cytostatic/cytotoxic cytokines such as the interferons and tumor necrosis factors, and antibody-dependent cell-mediated cytolysis. We have shown that IL-12 is capable of enhancing several of these antitumor mechanisms. IL-12 can enhance the lytic activity of NK/LAK cells and can facilitate specific CTL responses. In addition, IL-12 is a growth factor for both activated NK cells and for activated T cells, including $CD8^+$ CTL. IL-12 can also stimulate the production of IFN-γ and TNF-α. Recently, Lieberman *et al.* (13) reported that IL-12 could augment antibody-dependent cell-mediated lysis of human colon carcinoma cells in culture. Possible effects of IL-12 on monocyte-mediated tumor cell lysis have not yet been examined.

The potential utility of cytokines in the treatment of tumors has been demonstrated in studies using IL-2. Administration of IL-2 has been shown to cause regression of established tumors in both experimental animals (16) and in man (17, 18). However, the clinical use of IL-2 has been limited by the severe toxicities associated with its administration (17, 18). The properties of IL-12 that have been delineated to date suggest that it may not be as toxic as IL-2 when administered *in vivo*. In part, the toxic effects of IL-2 appear to be associated with a massive proliferation of NK cells which subsequently infiltrate into normal tissues such as lung and liver and cause tissue damage (9, 15). However, the mitogenic activity of IL-12 for resting NK cells is much less than that of IL-2. In addition, the production of TNF in response to IL-2 appears to play a central role in mediating some aspects of IL-2 toxicity (3, 14). As noted above, the amount of TNF produced in response to IL-12 is much less than that elicited by IL-2. Because of the synergies that

have been observed between IL-12 and IL-2 (11, 12, 20), it may be that combining IL-12 with low concentrations of IL-2 would result in more efficacy with less toxicity than using either cytokine alone.

The possible antitumor effects of IL-12 must next be tested in animal (rodent) tumor models. Unfortunately, human IL-12 has been found to be ineffective in enhancing the lytic activity of mouse NK cells or in stimulating the proliferation of activated mouse T cells (M. Gately, unpublished results). Recently, we have succeeded in cloning the cDNAs encoding the two subunits of mouse IL-12 (D. Schoenhaut, A. Chua, A. Wolitzky, *et al.*, manuscript in preparation). Efforts are in progress to express and purify sufficient amounts of mouse IL-12 for *in vivo* testing. These efforts should soon make it possible to assess *in vivo* the potential applications of IL-12 as an antitumor agent.

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structure-activity correlation with the ability of the esters to stimulate the translocation of PKC from the cytosolic to the particulate fraction. Such an antigen-induced translocation in the region of CTL- target contact would provide a region of specialized membrane necessary for the initiation of the specific and unidirectional delivery of the "lethal hit".

3.23.6

CHARACTERIZATION OF A HUMAN CTL MATURATION FACTOR.
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Previously we have shown that IL-2-depleted supernatants from either human mixed leukocyte cultures or PHA-activated human leukocyte cultures contained a factor(s) capable of synergizing with human recombinant IL-2 (rIL-2) in promoting specific cytolytic T lymphocyte (CTL) responses to allogeneic, uv-irradiated melanoma cells *in vitro*. We tentatively called this late-acting factor CTL maturation factor ($T_cMF$).

In the present study, $T_cMF$ was found to be both trypsin- and pronase-sensitive but neuraminidase- and RNase-insensitive, suggesting that $T_cMF$ is a protein. When chromatographed on a Sephacryl S-200 column, $T_cMF$ activity was recovered in two peaks: a 55-65 kD peak, which was observed even when 4 M urea was included in the column buffer, and a 15-20 kD peak. In additional experiments we examined the relationship of $T_cMF$ to other cytokines which may play a role in CTL responses. Neither recombinant human gamma interferon (rIFN-$\gamma$) nor rIFN-$\alpha$ over a wide range of concentrations displayed any $T_cMF$ activity. Furthermore, a neutralizing monoclonal anit-IFN-$\gamma$ did not neutralize $T_cMF$ activity. Likewise, recombinant human IL-1$\alpha$ at concentrations equivalent to the IL-1 activity in the culture supernatants possessed minimal $T_cMF$ activity. Thus $T_cMF$ appears to be distinct from both interferon and IL-1$\alpha$.